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Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

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Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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ISOLATION OF PRECURSOR CELLS AND THEIR USE FOR TISSUE REPAIR.

The present invention relates to the field of tissue engineering in general, and more specifically to the identification of skeletal precursor cell populations for the repair of connective tissue *in vivo*.

BACKGROUND OF THE INVENTION

Cartilage is a tissue composed by a cellular component, chondrocytes, and by an extracellular matrix typically rich in type II collagen and highly sulfated proteoglycans. The latter property confers cartilage its peculiar histochemical characteristics that are strong staining with alcian blue at low pH (0.2-2.5) and metachromacy with toluidin blue and safranin O. The abundance of type II collagen, link protein, and proteoglycan aggrecan, together with the presence of minor collagens such as type IX and type XI collagen are hallmarks of cartilage tissue.

In post-natal mammals cartilage contributes to the structure of several organs and systems like the articular surface of diarthrodial joints and other joint-associated structures such as menisci, the ear, the nose, the larynx, the trachea, the bronchi, structures of the heart valves, part of the costae, synchondroses, entheses etc. In some of the mentioned locations (e.g. entheses, the annulus fibrosus of the intervertebral disks, the menisci, insertion of ligaments etc.) for the abundance of collagens (mostly type I collagen) it is called *fibrocartilage*. In other locations (e.g. the pinna of the ear, epiglottis etc.) it is particularly rich of elastin and is called *elastic cartilage*. In all the other structures including articular cartilage, for its semi-transparent, clear aspect it is called *hyaline cartilage*.

In embryonic development mesenchymal cells aggregate and differentiate to form cartilage anlagen, which provide the mold of the future long bones. These cartilage templates in dev lopment evolve undergoing endochondral bone formation through a cascade of events including chondrocyte hypertrophy, vascular invasion, mineralization, and eventually replacement by bone, except

for a thin layer at the extremities of the bone elements that will differentiate into the articular surface of dyarthrodial joints. In this locations cartilage tissue remains hyaline for all the life-span of the individual. With aging, articular cartilage is well known to undergo a process of senescence, being affected in its mechanical properties and intrinsic resilience.

The current therapy for loss of cartilage tissue is replacement with a prosthetic material such as silicone for cosmetic repairs, or metal alloys for joint refinement. Placement of prosthetic devices, however, is a very artificial way of repairing, usually associated with loss of underlying bone without recovery of the full function allowed by the original cartilage tissue. Serious long-term complications associated with the presence of a permanent foreign body can include infection, erosion and instability. Implantation of sterilized bone or bone powder with surgical steel seeded with bone cells has been largely unsuccessful because of the non-degradable nature of the cell support. U.S. Pat. No. 4,609,551 discloses that fibroblasts exposed *in vitro* for at least three days to a soluble bone protein are capable of stimulating a chondrogenic response *in vitro* and/or *in vivo*. The activated fibroblasts are then transferred *in vivo* by combining them with a biodegradable matrix, or by intra-articular injection or attachment to allografts and prosthetic devices. The disadvantage of this method is that chondrogenesis is not allowed to develop in the short-term cultures and there is an unduly heavy reliance on the exposed fibroblasts at the implant site for cartilage synthesis. EP-A-739,631 discloses poducing a biological material comprising reconstituted cartilage tissue by growing chondrocytes on a flexible sheet of 1.5 mm thick demineralized natural bone. This however will be useful only when the bone is not self-derived because harvesting self-derived bone requires a complicated and painful surgery.

Joint surface defects can be the result of various etiologies such as inflammatory processes, neoplasias, post-traumatic and degenerative events, etc. Whatever the cause, due to its limited capacity for repair, cartilage heals poorly with, at b st, some scar formation or fibrocartilaginous tissue. This partial repair of the articular surface leads to osteoarthritis and severe functional

disability. Based on the depth of the injury, two types of joint surface defects are defined, the osteochondral (or full-thickness) and the superficial (or partial-thickness).

Osteochondral (or full-thickness) joint surface defects include damage to the articular cartilage, the underlying subchondral bone tissue, and the calcified layer of cartilage located between the articular cartilage and the subchondral bone. They typically arise during severe trauma of the joint or during the late stages of degenerative joint diseases, e.g. during osteoarthritis. Since the subchondral bone tissue is both innervated and vascularized, damage to this tissue may be painful. Osteochondral defects rely on the extrinsic mechanism for repair. Extrinsic healing relies on mesenchymal elements from subchondral bone to participate in the formation of new connective tissue. This repair tissue may undergo metaplastic changes to form fibrocartilage that does however not display the same biochemical composition or mechanical properties as normal articular cartilage or subchondral bone and degenerates with use.

Superficial or partial-thickness injuries of the articular cartilage that do not penetrate the subchondral bone rely on the intrinsic mechanism for repair. Soon after superficial injury, chondrocytes adjacent to the injured surfaces show a brief burst of mitotic activity associated with an increase in metabolic activity and matrix synthesis. Despite these attempts at repair, there is no appreciable increase in the bulk of cartilage matrix and the repair process is rarely effective in healing the defects. Although initially sometimes painless, partial-thickness defects often degenerate into osteoarthritis of the involved joint.

Repair of articular cartilage defects with suspensions of chondrocytes has been carried out in a variety of animal models and is now employed in humans (Brittberg M. et al., *N. Eng. J. Med.* 1994, 331:889-95). Autologous chondrocytes obtained from an unaffected area of the joint are released, expanded *in vitro* in the presence of autologous serum and subsequently injected under a periosteal flap sutured to cover the cartilage defect. This procedure has led to a proven at least symptomatic amelioration. This promising approach has still wide margins

for improvement, since it is known that *in vitro* expansion of chondrocytes results, after a limited number of cell divisions, in a loss of their phenotypic stability (as defined by the ability of chondrocytes to form hyaline cartilage in vivo) making the cell suspension to be injected unreliable.

Three alternative approaches have been developed in an attempt to improve the success rate in treating mammalian articular cartilage defects. In the first approach, synthetic carrier matrices are impregnated with chondrocytes and then implanted into the cartilage defect where they hopefully produce and secrete components of the extracellular matrix to form articular cartilage at the site of the defect. A variety of synthetic carrier matrices have been used to date and include three-dimensional collagen gels (e.g. U.S. Pat. No. 4,846,835), reconstituted fibrin-thrombin gels (e.g. U.S. Pat. Nos. 4,642,120; 5,053,050 and 4,904,259), synthetic polymer matrices containing polyanhydride, polyorthoester, polyglycolic acid and copolymers thereof (U.S. Pat. No. 5,041,138), and hyaluronic acid-based polymers. Once a mitotically expanded population of chondrocytes is obtained, the cells can be implanted either back into the same subject from which their parent cells were originally derived (autologous implantation), or into a different subject (heterologous implantation). In addition, heterologous implantation may use chondrocytes obtained from a related or unrelated individual of the same species (allogeneic), or from a different species (xenogeneic). Alternatively, chondrocytes may be obtained from an established, long-term cell line that is either allogeneic or xenogeneic.

Autologous implantation requires that chondrocytes are harvested from an uninvolved area of the joint surface from the patient and then in vitro culture expanded to sufficient number or density for an effective implant. The amount of time required for such sufficient expansion, however, may preclude the effective use of an autologous culture since some cartilage repairs should be carried out immediately or within a short time after a traumatic injury occurs. Another limitation is the mitotic potential of the cells, since there is a limitation to the numb r of times the cells can b expanded, and the ultimate quantity of cells produced for therapy may be limited. In addition, where a severe debilitating joint disorder causes general degradation of cartilage tissue throughout a patient's

body, namely in elderly people, there may be very little unaffected cartilage tissue available from which to initiate a chondrocyte culture. The introduction of heterologous chondrocytes into a patient, on the other hand, may stimulate an undesirable immune response directed against the implanted material, leading to potential rejection of the newly formed and engrafted cartilage tissue. In addition, heterologous implantation risks the transmission to the subject of infectious agent(s) present in the tissue or cell line.

Moreover, when using synthetic carrier matrices neo-cartilage may be formed around the periphery of the implant thereby preventing integration of the implant into the cartilage defect. Monitoring the formation and development of the resulting synthetic cartilage in situ is difficult to perform and usually involves an arthroscopic or open joint examination. Furthermore, implants containing synthetic polymer components may be unsuitable for repairing large cartilage defects since polymer hydrolysis in situ inhibits the formation of cartilage and/or its integration into the defect.

In the second approach, the defect is filled with a biocompatible, biodegradable matrix containing chemotactic and mitogenic growth factors to stimulate the influx of chondrocyte progenitor cells into the matrix in situ. The matrices optimally contain pores of sufficient dimensions to permit the influx into, and proliferation of the chondrocyte progenitors within the matrix. The matrix also may contain growth factors to stimulate the differentiation of chondrocyte progenitor cells into chondrocytes which in turn secrete extracellular matrix components to form cartilage at the site of the defect in situ (e.g. U.S. Pat. Nos. 5,206,023 and 5,270,300 and EP-A-530,804). This approach however results in problems similar to those associated with the first approach hereinabove. Furthermore there is no data so far that articular cartilage contains chondrocytic progenitors for partial-thickness defects.

In the third approach, chondrocytes may be cultured and expanded in vitro to form synthetic cartilage-like material that is implanted subsequently into the cartilage defect. This has the advantage over the previous methods in that the development of the synthetic cartilage material may be monitored, through

biochemical and morphological characterization, prior to implantation. Growing chondrogenic cells may b achieved in either an anchorage-dependent or an anchorage-independent manner. In the latter, chondrogenic cells may be cultured as colonies within an agarose gel. Heretofore, only small pieces of cartilage tissue of undefined shape have been prepared using this manner. Furthermore, the resulting cartilage remains embedded within a gel matrix making it less suitable for implantation into mammals. Alternatively, in another anchorage-independent method, chondrocytes may be cultured as colonies in suspension culture. However the resulting particles containing synthetic cartilage-like material are usually small and of undefined shape thus making them unsuitable for implantation and repair of a predetermined articular cartilage defect. This would rather result in several little pieces of cartilage, completely separated from each other, and far from being very well integrated among them and the surrounding cartilaginous tissue.

In the anchorage-dependent method, primary cultures of chondrocytes isolated from primary tissue are grown as monolayers attached to the surface of a cell culture flask (e.g. U.S. Pat. No. 4,356,261). The primary cells derived directly from explant tissue remain capable of producing and secreting extracellular components characteristic of natural cartilage, specifically type II collagen and sulfated proteoglycans. However, it is well known that during *in vitro* expansion procedures chondrocytes in monolayer undergo a dedifferentiation process, thereby losing their ability to organize hyaline cartilage in vivo. Therefore, until now it has not been possible to prepare large patches of articular cartilage from small pieces of biopsy tissue using the anchorage-dependent procedures of U.S. Pat. No. 4,356,261.

In order to solve the above problems, U.S. Pat. No. 5,723,331 provides a method for preparing in vitro large quantities of synthetic cartilage from small samples of biopsy tissue which, based on the discovery that chondrogenic cells may be isolated from a variety of tissues, e.g. pre-existing cartilage, perichondrial tissue or bone marrow, and expanded in vitro prior to cartilage formation, includes first seeding denuded (i. . isolated from an enzymatically or mechanically disaggregated tissue) chondrogenic cells, proliferated *ex vivo*, into a pre-shaped well having a cell contacting, cell adh sive surface, and then

culturing the proliferated chondrogenic cells in the well for a time sufficient to permit the cells to secrete an extracellular matrix th reby to form a three-dimensional, multi cell-layered patch of synthetic cartilage.

A further disadvantage of these methods is that the chondrocytes must be obtained from the patient, typically by a biopsy, culture expanded, and then implanted on a matrix. This is relatively easy in laboratory animals, but presents greater logistical problems in humans where a defect is created by the biopsy required to provide cells for repair of another defect. Moreover, if the defect includes a part of the underlying bone, this is not corrected using chondrocytes, which are already differentiated and will not form new bone. The bone is required to support the new cartilage.

The use of mesenchymal stem cells has also been proposed for the repair of many tissues including cartilage. Mesenchymal stem cells are a potential alternative source of cartilage-producing cells. They are generally recognized as pluripotent cells which are capable of dividing many times to produce progeny cells that can eventually give rise to connective tissues, including cartilage, bone, tendons, ligaments, marrow stroma. By definition, they are not limited to a fixed number of mitotic divisions.

Stem cells are defined as cells that are undifferentiated, which can divide without limit to yield cells that are either stem cells or cells that further differentiate to yield different types of progenitor cells, including mesenchymal stem cells. Those mesenchymal stem cells are pluripotential cells that are capable of differentiating into any of the specific types of mesenchymal or connective tissues, including skeletal tissues. Mesenchymal stem cells were isolated from bone marrow or other sources such as periosteum, placenta, umbilical cord, skin, and blood (e.g. in U.S. Pat. No. 5,811,094). Pluripotent mesenchymal stem cells have also been isolated from muscle (Patè et al., *Proc. 49th Ann. Sess. Forum Fundamental Surg. Problems* Oct. 1993, 587-9), heart (Dalton et al., *J. Cell Biol.* 1993, 119 R202) and granulation tissue (Lucas et al., *J. Cell. Biochem.* 1993, 122 R212). Pluripot ncy was demonstrated using a non-specific inducer, d xamethason , which elicits differentiation of the stem cells

into chondrocytes (cartilage), osteoblasts (bone), myotubes (muscle), adipocytes (fat), and connective tissue cells.

Unfortunately, although it is highly desirable to have stem cells which are easily obtained by a muscle or a skin biopsy, cultured to yield large numbers, and then used as a source of chondrocytes or osteoblasts or myocytes, there is no known specific inducer of the mesenchymal stem cells that yields only cartilage. In vitro studies in which differentiation is achieved yield a mixture of cell types. In U.S. Pat. Nos. 5,226,914 and 5,197,985 the cells were seeded into porous ceramic blocks and, subcutaneously implanted into nude mice, yielded primarily bone. However, U.S.Pat. No. 5,906,934 discloses that under very specific conditions mesenchymal stem cells in a suitable polymeric carrier (such as polyglycolic acid mesh) implanted into a cartilage and/or bone defect will differentiate to form cartilage and/or bone, as appropriate. Also U.S. Pat. No. 5,919,702 discloses chondrocyte progenitor cells isolated from umbilical cord sources, e.g. from Wharton's jelly, and cultured so as to give rise to chondrocytes that can produce cartilage tissue. Also in another attempt to avoid the drawbacks of current cartilage and bone repair techniques which cause bleeding and involve the use of mechanically weak non self-derived material, U.S. Pat. No. 5,866,415 suggests treating cartilage or bone defects with a biological material obtained by attaching in vitro cartilage or bone forming cells to a periosteum of sufficient size to accomodate the defect.

Figure 1 shows schematically the hierarchical cascade of cells in the differentiation process, starting from the undifferentiated mesenchymal stem cells downwards to the fully differentiated cells of the skeleton. U.S. Pat. No. 5,811,094 describes methods to identify, selectively isolate and enrich by culture expansion mesenchymal stem cells. Said patent does not provide methods for isolating, purifying, and culturally expanding skeletal precursor cells, methods which are the purpose of the present invention. Our efforts are focused on the skeletal precursor cells, as hereinafter defined, unraveling the molecular cascade of events underlying the differentiation pathways leading to the specialized cells of the skel tal tissues, with specific att ntion to the generation of the stable

chondrocyte.

Transforming growth factor-.beta ("TGF-β") refers to a family of related dimeric proteins, which regulate the growth, and differentiation of many cell types. Members of this family include TGF-β 1, TGF-β 2, TGF-β 3, TGF-β 4, TGF-β 5, morphogenic proteins ("MP") such as MP-121 and MP-52, inhibins/activins (such as disclosed in EP-A-222,491), osteogenic proteins ("OP"), bone morphogenetic proteins (hereinafter denoted "BMP"), growth/differentiation factors ("GDF") such as GDF-5, GDF-6, GDF-9 and Nodal. TGF-β was first characterized for its effects on cell proliferation. It both stimulated the anchorage-independent growth of rat kidney fibroblasts and inhibited the growth of monkey kidney cells. TGF-β family members have been shown to hav many diverse biological effects, e.g. they regulate bone formation, induce rat muscle cells to produce cartilage-specific macromolecules, inhibit the growth of early hematopoietic progenitor cells, T cells, B cells, mouse keratinocytes, and several human cancer cell lines. TGF-β family members increase the synthesis and secretion of collagen and fibronectin, accelerate healing of incisional wounds, suppress casein synthesis in mouse mammary explants, inhibits DNA synthesis in rat liver epithelial cells, stimulate the production of BFGF binding proteoglycans, modulate phosphorylation of the epidermal growth factor ("EGF") receptor and proliferation of epidermoid carcinoma cells and can lead to apoptosis in uterine epithelial cells, cultured hepatocytes and regressing liver. TGF-βs can mediate cardio-protection against reperfusion injury by inhibiting neutrophil adherence to endothelium and it protects against experimental autoimmune diseases in mice. On the whole, proteins of the TGF-β family are multifunctional, hormonally active growth factors and also have related biological activities such as chemotactic attraction of cells, promotion of cell differentiation and tissue-inducing capabilities. Differences in their structure and their affinity for receptors lead to considerable variations in their exact biological function.

In contrast to the foregoing reports of th ability of TGF-β to induce the production of cartilage-sp cific macromolecul s in muscle cells and

chondrocytes, TGF-β was found to act synergistically with fibroblast growth factor to inhibit the synthesis of collagen type II by chicken sternal chondrocytes and in rat chondrocytes. In fact, TGF-β has emerged as the prototypical inhibitor of the proliferation of most normal cell types in vitro as well as in vivo, exhibiting a remarkable diversity of biological activity. TGF-β 1 has been purified from human and porcine blood platelets and recombinant TGF-β 1 is currently available.

Among the sub-family of BMPs, the structures of BMP-1 through BMP-13 have previously been elucidated. The unique inductive activities of these proteins, along with their presence in bone, suggests that they are important regulators of bone repair processes and may be involved in the normal maintenance of bone tissue. Recently, the BMP-12-related subfamily of proteins, including BMP-13 and MP52 (see e.g. WO93/16099 and U.S. Pat. No. 5,658,882), was shown to be useful in compositions for the induction of tendon/ligament-like tissue formation and repair. U.S. Pat. No. 5,902,785 discloses that BMP-12 related proteins are particularly effective for the induction of cartilaginous tissue and that BMP-9 is useful for increasing proteoglycan matrix synthesis and therefore for the maintenance of cartilaginous tissue. It also describes compositions comprising a BMP-12 related protein and additionally including one or more TGF-β proteins proven to be osteogenic, preferably BMP-2, -4, -5, -6 and/or BMP-7 as useful for the regeneration of multiple tissue types (for example at the interface or junction between tissues) and especially useful for the treatment of articular cartilage, in which the articular surface, cartilage, subchondral bone and/or tidemark interface between cartilage and bone need to be repaired. The same patent further describes compositions comprising a BMP-12 related protein together with a protein useful for the maintenance of chondrocytes or cartilaginous tissue such as BMP-9, the said compositions being especially useful for the induction and maintenance of cartilaginous tissue at a site in need of cartilage repair such as an articular cartilage defect.

WO96/14335 discloses, using mRNA prepared from newborn articular cartilage, the isolation of two members of the BMP family, designated Cartilage-

derived morphogenetic proteins-1 and -2 (CDMP-1, CDMP-2). More specifically, WO96/14335 discloses a purified cartilage extract that stimulates local cartilage formation when combined with a matrix and implanted into a mammal, said extract being produced by obtaining cartilage tissue, homogenizing said cartilage tissue in the presence of chaotropic agents under conditions that permit separation of proteins from proteoglycans, separating said proteins from said proteoglycans, and obtaining said proteins. It also discloses an isolated DNA molecule encoding a protein having chondrogenic activity *in vivo* but substantially no osteogenic activity *in vivo*. The role of CDMP-1 as a regulator of skeletal growth is now well documented. Storm et al. (1994) in *Nature* 368, 639-43 and Chang et al. (1994) in *J.Biol.Chem.* 269, 28227-34 independently established that CDMP-1 mapped close to the brachypodism locus on chromosome 2 in mice and might be involved in the brachypodism phenotype. Also the expression patterns of CDMPs suggests an important role for these genes in joint morphogenesis. WO98/59035 also discloses a method of maintaining a cartilaginous phenotype in chondrocytes *in vitro*, comprising culturing the chondrocytes in a serum-free medium containing such a CDMP and/or BMP. Table 1 below summarizes the BMP superfamily members in mammals (Reddi AH, *Nature Biotechnol.* 1998, 16: 247-52).

Table 1

BMP subfamily	Generic name	BMP designation
BMP 2/4	BMP-2A	BMP-2
	BMP-2B	BMP-4
BMP 3	Osteogenin	BMP-3
	Growth/differentiation factor 10	BMP-2B
Op-1/BMP-7	BMP-5	BMP-5
	Vegetal related-1 (Vgr-1)	BMP-6
	Osteogenic Protein-1 (Op-1)	BMP-7
	Osteogenic Protein-2 (Op-2)	BMP-8
	Osteogenic Protein-3 (Op-3)	BMP-8B
	Growth/differentiation factor 2 (GDF-2)	BMP-9
	BMP-10	BMP-10
	Growth/differentiation factor 11 (GDF-11)	BMP-11
GDF-5,6,7	Growth/differentiation factor 7 (GDF-7) or cartilage-derived morphogenetic protein-3 (CDMP-3)	BMP-12
	Growth/differentiation factor 6 (GDF-6) or cartilage-derived morphogenetic protein-2 (CDMP-2)	BMP-13
	Growth/differentiation factor 5 (GDF-5) or cartilage-derived morphogenetic protein-1 (CDMP-1)	BMP-14
	BMP-15	BMP-15

Other families of growth factors have been shown to be involved in cartilage differentiation and maintenance such as the fibroblast growth factors

(FGFs), which are a family of polypeptid growth factors involved in a variety of activities. One of their receptors, FGF receptor 3 (FGFR3) (Keegan K. et al., *1991* Proc. Nat. Acad. Sci. 88: 1095-99), is known to play a crucial role in chondrogenesis. Point mutations in the fgfr3 gene resulting in a ligand-independent constitutively active protein (which means that the FGF signaling is always active also in the absence of the ligand) cause skeletal abnormalities as achondroplasia and thanatophoric dysplasia.

As already outlined in page 2, although autologous chondrocyte transplantation ("ACT") is becoming a widely accepted technique for repair of joint surface defects ("JSD"), it still presents some drawbacks. More in detail, this procedure implies *in vitro* expansion - in the presence of autologous serum - of autologous chondrocytes obtained from an uninvolved area of the joint, followed by the implantation of the chondrocyte suspension under a periosteal flap sutured to seal the joint surface defect. Cell expansion is necessary to obtain from a small cartilage biopsy a number of cells sufficient to repair the cartilage defect. To date, however, it is not known to expand chondrocytes without hampering their phenotypic stability. Indeed it is well known, as explained before, that *in vitro* expansion of chondrocytes results in cell de-differentiation. This implies that chondrocyte expansion pays the price of loss of phenotypic stability and therefore the need of a quality control on expanded chondrocytes to be used for ACT. At the end of cell expansion the chondrocyte population is composed of some cells that retain their phenotypic stability, and others that still can proliferate but will not anymore contribute to cartilage repair. In order to obtain a consistent cell suspension for ACT, it is desirable to select stable chondrocytes within the expanded cell population. Chondrocytes are skeletal cells able to grow in anchorage-independent agarose cultures. The ability of chondrocytes to grow in anchorage-independent conditions is critical for those cells to survive and organize cartilage tissue once injected as a cell suspension for repair of JSD, but is probably not the only necessary phenotypic trait.

Therefore there is a need in the art for identifying and s lecting an easily accessible and expandable source of pluripotent sk l tal precursor cells. There

is a need in the art for solving the various problems encountered in the cartilage repair known methods. There is also a need in the art for developing repair techniques for connective tissues including cartilage, e.g. for medical problems such as rheumatoid arthritis and osteoarthritis. This is indeed a highly relevant clinical problem since more than one million bone-grafting procedures are performed annually in the world. There are a number of suggestions in the prior art that some mesenchymal stem cells could specifically yield cartilage or, as needed, other connective tissues. For instance bone marrow contains populations of pluripotent mesenchymal stem cells having the capacity to differentiate into a wide range of cell types of the mesenchymal, hematopoietic and stromal lineages. It is also known that mesenchymal stem cells cultured *in vitro* can be induced to differentiate into bone or cartilage *in vivo* and *in vitro*, depending upon the tissue environment or the culture medium into which the cells are placed. To date, however, very few common cell markers or differentiation antigens were identified. Examples of such markers include Ly-6 antigens for murine osteoblasts (see Horowitz et al. (1994) in *Endocrinology*, 135, 1032-43) and CD34 for human hematopoietic cell types. On the other hand, periosteum and marrow are known as the most common sources of precursor cells having osteogenic potential. More specifically it has been shown that cells from marrow, when isolated and expanded by the culture method of Friedenstein, will form bone, cartilage and fibrous tissue when implanted. However Friedenstein in *Calcif.Tiss.Int.* (1995) 56(S):S 17 admitted that obstacles, such as the need for culturing for several passages and developing a method for transplanting such cells, must be overcome before clinical utility of this discovery can be confirmed. Therefore there is a need in the art for the proper identification of pluripotent skeletal precursor cells in a wide range of easily accessible and expandable sources. These goals and other purposes are achieved by means of the following objects of the present invention.

SUMMARY OF THE INVENTION

A first object of the present inv ntion is the identification and characterization of precursor cells in a wide range of easily accessibl and expandabl sources. The precursor cells may be skeletal precursor cells such

as, among others, periosteum, bone marrow and synovial membrane, while using a set of molecular markers. A second object of th present invention is the use of such precursor cells and molecular markers for the repair of a wide rang of connective tissues. A third object of the present invention is the use of such precursor cells as a source of transforming growth factors ("TGF") linked to the phenotypic stability of a certain cell population involved in a certain differentiation pathway, such as for instance members of the TGF-β family which are positively associated with chondrocyte phenotypic stability. A fourth object of the present invention is the use of such precursor cells and molecular markers as matrix producing cells in tissue engineering procedures. A fifth object of the present invention is the co-implantation of expanded skeletal precursor cells and chondrocytes for *in vivo* cartilage repair.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of the hierarchical cascade of cells in the differentiation pathways.

Figure 2 is a picture showing that skeletal precursor cells express CDMP-1.

Figure 3 consists of two histological pictures showing that skeletal precursor cells can grow anchorage-independently retaining their phenotypic stability *in vivo*.

Figure 4 is a picture showing that the implant retrieved from mouse muscle is formed by human cells.

Figure 5 is a set of two histological pictures showing cartilaginous tissue obtained *in vitro* from human skeletal precursor cells (5B).

DETAILED DESCRIPTION OF THE INVENTION

Terms used throughout this disclosure are defined as follows:

Chondrocyte stability

The capacity of a cell suspension (either obtained from cartilage tissue or from any other tissue containing cells with chondrog nic potential) to produce a hyaline cartilag implant without signs of vascular invasion or endochondral bon

formation, 3 weeks after its injection into a mammal.

Chondrogenic

The capacity to promote or stimulate cartilage growth, as applied to cells such as chondrocytes and to cells which themselves differentiate into chondrocytes.

Connective tissue

Any structural tissue in the body of a mammal including bone, cartilage, ligament, tendon, meniscus and joint capsule.

Differentiation

A biological process by which primitive unspecialized cells undergo a series of cellular divisions giving rise to progeny having more specialized function(s). Terminal differentiation provides a highly specialized cell having unique functional and phenotypic characteristics.

Mesenchymal stem cell

A primitive cell type having the capacity for self-regeneration and for differentiation through a series of lineages to produce progeny cells with wide phenotypic variety including connective tissue, marrow stroma, adipocytes, dermis and muscle.

Osteogenic

The capacity to promote or generate the production of bone.

Phenotypic stability

The capacity of any cell to reorganize *in vivo* the structure of a specific tissue, either the original tissue where the cells were taken from, or a different tissue the cells have been forced to form under specific conditions.

Precursor cell

A cell having the capacity of undergoing differentiation to perform a

specific function.

Skeletal precursor cell

A cell no longer undifferentiated, but already committed towards any of the differentiation pathways of the skeletal tissues.

The present invention will mainly be described with reference to skeletal precursor cells but the invention is not limited thereto. The present invention relates to the use of embryonic markers which identify that certain precursor cells have entered a post-natal differentiation pathway. It is believed that the present invention is not limited in any way as to cell type provided they are associated with organism with differentiated cells. Examples are animals, especially mamalls, insects, plants. The present invention is particularly useful iwith respect to mamallian precursor cells, in particular, skeletal precursor cells, more inparticular skeletal precursor cells of humans and horses but it is not limited thereto. The present invention makes use of cell embryonic markers which are considered to be available in or on all differentiated cells or precursor cells of such differentiated cells in any differentiated life form. Such embryonic markers are considered to be a necessary part or associated with a necessary part of embryogenesis as the growing organism during differentiation has also the necessity of identifying differentiated or partly differentiated cells and this must be achieved biochemically. Hence, the present invention has wide application.

The present invention is based upon surprising discoveries which have general relevance to the development of all differentiated lif-forms in particular mamals such as humans or horses.

First, pluripotent human skeletal precursor cells can be reliably identified by a set of molecular markers. Secondly, such skeletal precursor cells upon consistent and proper conditions are able to produce and repair various connective tissues including cartilage, when used either alone or in association with chondrocytes. And thirdly, such skeletal precursor cells can be induced to xpress genes linked to specific tissues.

We provide evidence that th expression of CDMP-1 qualifies a certain culture expanded cell population as skeletal precursor cells. This is an

unexpected result, since CDMP-1 has always been known to promote chondrogenic differentiation and never linked to the phenotype of skeletal precursor cells. Regardless the source, cells are culture expanded and assessed by RT-PCR analysis for the expression of CDMP-1. Only the CDMP-1 expressing cells can be successfully processed to be directed into a specific differentiation pathway of any skeletal connective tissue, including cartilage. Interestingly, whenever a skeletal precursor cell as defined by the expression in RT-PCR of CDMP-1, undergoes differentiation such as towards the chondrocytic phenotype, entering a specific differentiation pathway is always preceded by the downregulation of the expression of CDMP-1.

A first embodiment of the present invention consists of the use of cartilage-derived morphogenetic protein CDMP-1 or a transforming growth factor having at least 80% homology with CDMP-1 as a marker of skeletal precursor cells from any part of a mammalian body. In other words, regardless of the source of cells, CDMP-1 or a homolog thereof is selectively expressed by skeletal precursor cells, and is downregulated as soon as said skeletal precursor cells undergo a differentiation step towards any mature lineage. For instance, when skeletal precursor cells differentiate into chondrocytes, the expression of cartilage markers such as type II collagen, type IX collagen, or type XI collagen, is always preceded by the disappearance of CDMP-1. This first embodiment is based upon the characterization of human skeletal precursor cells from various-aged donors (ranging from 18 to 78 years old) over a large number of passages by molecular markers. Using reverse transcriptase polymerase chain reaction ("RT-PCR") analysis, it was observed that skeletal precursor cells are phenotypically stable through serial passaging, retaining their phenotype and their potential of differentiation even after having been frozen in liquid nitrogen for several months.

A second embodiment of the present invention consists of further using reagents and/or antibodies recognizing specific cell surface markers for sorting, out of th mammalian skeletal precursor c ll pool, cell subpopulations for further processing by proper treatment towards specific lin ages. Such enrichment

protocols will avoid the unpleasant eventuality of other contaminating tissues arising from the pool of skeletal precursor cells. Once enriched, those cells can be directed to any differentiation pathway such as the chondrogenic pathway, by culturing under consistent and appropriate conditions with or without morphogens/growth factors to end up with a homogeneous cell population, such as chondrocytes with a phenotypic stability. In particular, we demonstrate that periosteum, bone marrow, and synovial membrane contain CDMP-1 expressing skeletal precursor cells that can be committed towards chondrogenesis using appropriate culture conditions. In contrast to previous studies of Nakahara et al. (1991), *J. Orthop. Res.* 9:465-76, we observed that independently of the age, human skeletal precursor cells are easily accessible and expandable and can be induced to differentiate into chondrocytes.

A third embodiment of the present invention is the use of the CDMP-1 marked skeletal precursor cells for producing or repairing connective tissue in general, including trachea, cardiac valves, vocal cords and the like. This further use is based on the capacity of such skeletal precursor cells to retain the intrinsic potential of multilineage differentiation, which make them good candidates for tissue engineering protocols. Isolation, expansion and sorting cell populations using the specific markers of the invention lead to the proper cell pool suitable for such repair procedures. More specifically, using CDMP-1 marked skeletal precursor cells we have developed proper culture conditions leading to the induction and formation of cartilage *in vitro*. It is important to note that treatment of skeletal precursor cells in monolayer culture with various growth factors did not result in any response in terms of osteogenesis and/or chondrogenesis, as assessed by RT-PCR analysis for bone and cartilage markers, by alcian blue and von Kossa staining, and by alkaline phosphatase activity. For successful induction of cartilage, it appears necessary to culture CDMP-1 marked skeletal precursor cells at a very high cell density, for instance a cell density of at least 10^5 cells/ml, and preferably in the so-called micromass culture at a cell density of about 2×10^7 cells/ml. For successful induction of connective tissue, it is further advisable to add (*in vitro*) or administer (*in vivo*) a factor that stimulates differentiation of the skeletal precursor cells into the type of connective tissue to

be produced or repaired, e.g. a transforming growth factor-β such as TGF-β 1 or TGF-β 3, preferably at a rate of about 10 ng/ml in a chemically defined serum-free medium, to or together with the culture expanded CDMP-1 marked skeletal precursor cells.

A fourth embodiment of the present invention is the use of the CDMP-1 marked skeletal precursor cells as a source of growth factors, more specifically Transforming Growth Factors- β ("TGF-β") and Bone Morphogenetic Proteins ("BMP") known to be important in the stimulation and maintenance of the cartilage phenotype. This can help for instance for chondrocyte *in vitro* expansion, preventing chondrocytes from de-differentiation through serial passaging. This may be useful for the *in vitro* expansion procedure of human chondrocytes for transplantation using a medium derived from autologous skeletal precursor cells.

A fifth embodiment of the present invention is the use of CDMP-1 marked skeletal precursor cells as matrix producing cells. According to this embodiment, human skeletal precursor cells treated with appropriate growth factors (as in the third embodiment hereinabove) are able to produce extracellular matrix reminiscent of hyaline cartilage. The said treated cells can therefore be used as a matrix supply for the attachment and growth of chondrocytes in joint surface defects ("JSD") repair, and for tissue engineering procedures of the cartilaginous skeleton in general, e.g. for the treatment of subglottic stenosis, tracheomalacia, chondromalacia patellae, osteoarthritis and traumatic lesions, for instance using bio-resorbable polymers (such as polylactic acid or polyglycolic acid) locally applied to fill the lesion. In such a use, the said treated cells provide proper support for attachment and cell growth, eventually coated or mixed with growth factors. Such combinations of cells, polymer matrices and growth factors will also be useful in orthopedic reconstructive surgery. An alternative for this embodiment is a method for enhancing the implantation of a prosthetic device in conn ctive tissue comprising the step of implanting a prosthetic device having skeletal precursor cells adhered thereto under conditions suitabl for

differentiating the cells into the connective tissue desired.

A sixth embodiment of the present invention is the co-implantation of CDMP-1 marked skeletal precursor cells and chondrocytes for JSD repair and tissue engineering procedures of the cartilaginous skeleton in general. This embodiment is based on the surprising observation that the addition of expanded skeletal precursor cells to a chondrocyte suspension for cartilage repair has a dramatic impact on the ability of cartilage formation by chondrocytes, both *in vitro* and *in vivo*. The said co-implantation is able to substantially reduce the number of chondrocytes needed for successful JSD repair and it also results in a remarkable enhancement of the amount of cartilage produced. These observations will allow using freshly isolated chondrocytes instead of the impaired *in vitro* expanded chondrocytic cells, thus circumventing the problem related to the loss of cartilage-forming ability of chondrocytes resulting from *in vitro* expansion. As is well known in the art, *in vitro* cell expansion is in fact a critical step in autologous chondrocyte transplantation and can hamper the effectiveness of the injected cells in producing cartilage.

In addition to the numerous previously cited uses, the CDMP-1 marked skeletal precursor cells of the present invention have the advantage that they can be stored up to at least several months in cell banks under storage conditions including a storage temperature below –100°C, e.g. in liquid nitrogen, and in case of necessity thawed, properly treated, and implanted in the same individual at a site where new connective tissue is desired. This storage capability is surprising, since other cells including chondrocytes lose their phenotype when stored under the same conditions even for a short time.

The CDMP-1 marked skeletal precursor cells can also be used for heterologous transplantation in cases of HLA (Human Leukocyte Antigen) compatibility or for tissues where the risk of rejection is minimal and can be easily pharmaceutically prevented.

A more complete understanding of the present invention will be obtained by referring to the following illustrative examples.

Example 1 - Isolation of skeletal precursor cells from periosteum, synovial membrane, and bone marrow

Periosteum from various-aged human donors was aseptically harvested from the proximal medial tibia within 24 hours after death. Periosteum was rinsed twice with Hank's Balanced Salt Solution ("HBSS") available from Life Technologies, supplemented with antibiotic-antimycotic solution (100 units/ml penicillin, 100 µg/ml of streptomycin, and 0.25 µg/ml of amphotericin B, also available from Life Technologies), finely minced, and digested with 0.2% collagenase (Life Technologies) in high-glucose Dulbecco's Modified Eagle Medium ("DMEM")(Life Technologies) containing 10% fetal bovine serum (FBS) (available from Biowhittaker) and antibiotics. After overnight incubation at 37°C, periosteal cells were collected by centrifugation, washed twice, resuspended in high-glucose DMEM supplemented with 10% FBS and antibiotics, plated in a T25 culture flask, and allowed to attach for 4 days. After that period of time, adherent cells were washed by changing the medium.

Synovial membrane from various-aged human donors was aseptically harvested from the knee joints within 24 hours after death, and processed following the same protocol described above for periosteum.

Heparinized bone marrow samples from various-aged human donors were diluted with HBSS, layered onto Lymphoprep (1.077 g/ml, available from Nycomed, Oslo), and centrifuged at 300 x *g* for 20 minutes. Cells from the gradient interface were collected, washed three times in HBSS, and resuspended in medium for culture.

Example 2 – CDMP-1 as a marker of skeletal precursor cells

Cells isolated as described in example 1 from periosteum, synovial membrane, and bone marrow, were characterized by RT-PCR analysis as hereinafter described along with other cells such as human skin fibroblasts. CDMP-1 was found expressed only by skeletal precursor cells, and not by other cells. We furth r demonstrated that only the CDMP-1 marked culture expanded cells under specific conditions can differentiate .g. towards chondrogenesis,

while the CDMP-1 negative cells under the same conditions are not capable of undertaking any skeletal differentiation pathway. Furthermore, the appearance of cartilage markers is always preceded by the downregulation of the expression of CDMP-1.

Figure 2A shows RT-PCR analysis for the expression of CDMP-1 normalized to the expression of β-actin in different cell populations numbered from 1 to 4.

- Lane 1: human periosteum-derived skeletal precursor cells;
- Lane 2: human synovial membrane-derived skeletal precursor cells;
- Lane 3: human bone marrow-derived skeletal precursor cells;
- Lane 4: human skin fibroblasts.

Figure 2B shows RT-PCR analysis for CDMP-1 and type II collagen, normalized to the expression of β-actin, in human skin fibroblasts (lanes 1 and 2) and human periosteum-derived skeletal precursor cells (lanes 3 and 4) in micromass culture, either untreated (lanes 1 and 3) or treated for 6 days with 10 ng/ml TGF-β1 (lanes 2 and 4).

- Lane 1: human skin fibroblasts untreated;
- Lane 2: human skin fibroblasts treated with 10 ng/ml TGF-β1;
- Lane 3: human skeletal precursor cells untreated;
- Lane 4: human skeletal precursor cells treated with 10 ng/ml TGF-β1.

<u>RNA extraction and semi-quantitative RT-PCR analysis</u>

Total RNA was extracted and DNAse-treated from human cells using the S.N.A.P.™ Kit (available from Invitrogen). 1 μg of total RNA was reverse-transcribed to make cDNA with oligo(dT) primer using Thermoscript (Life Technologies). Polymerase chain reaction ("PCR") was performed in a volume of 10 μl adding 1μl out of 80 μl of the cDNA as a template, using Taq DNA polymerase (available from Eurogentech). When the sequence of the gene was known, primers were designed on different exons in order to distinguish cDNA from genomic DNA contamination. Before PCR analysis, cDNAs were equalized for the expression of the housekeeping gene β-actin. PCR for human β-actin was carried out stopping the reaction at each cycle starting from th 17th cycle in

order to make sure that PCR amplification was still in the linear phase. PCR products were electrophoresed in 1% agarose gel in TBE (Tris-borate/EDTA) electrophoresis buffer, stained with ethidium bromide, visualized by UV transillumination, and analyzed by densitometry using the Image Master software (available from Pharmacia-Biotech). cDNAs were diluted according to the relative intensity of the bands. To rule out that β-actin was differentially regulated in the different samples to be compared, the same analysis was also performed for the expression of another housekeeping gene, glucose-3-phosphate dehydrogenase (GAPDH). After equalization for β-actin and GAPDH, all samples were simultaneously tested for several genes, including the ones known to be involved in chondrogenesis and cartilage maintenance. For each gene, cycling was optimized in order that amplification was still in a linear phase when PCR was stopped for all samples.

Example 3 - *In vitro* cell expansion

Cells isolated according to example 1 were cultured in monolayer in high-glucose DMEM containing 10% FBS and antibiotics at 37°C in 95% humidified air and 5% CO_2, and the medium was replaced every 3 days. After 10 to 20 days of primary culture, when the sparsely attached cells reached confluence, they were washed twice with calcium and magnesium-free phosphate buffered saline (PBS) and harvested by treatment with trypsin-EDTA (0.25% trypsin, 1mM EDTA; Life Technologies), and replated by a 1:4 dilution for the first subculture. Cell passages were continued in the same way with a 1:4 dilution every 7-8 days when cells reached confluence.

Example 4 - Determination of skeletal precursor cell phenotypic stability through serial passaging

At each passage of the expansion procedure of example 3, skeletal precursor cells were harvested for total RNA extraction and RT-PCR analysis as above described. Their molecular profile stays stable through serial passaging, indicating that they can be largely expanded without hampering their property of skeletal precursors.

Example 5 - Anchorage-independent growth of skeletal precursor cells

The anchorage-independent growth of skeletal precursor cells from example 3 was assessed *in vitro* by agarose culture and *in vivo* by intramuscular injection into immunodeficient nude mice, as explained below. The injection of skeletal precursor cells into nude mice resulted into formation of a poorly differentiated, immature fibrocartilaginous tissue of human origin, as demonstrated by *in situ* hybridization for human-specific alu sequence.

Agarose culture

Agarose culture was performed according to the method of Benya et al., *Cell* (1982) 30:215-24. Briefly, 35 mm^2 Petri dishes were coated with 1% sterile high T_m agarose (available from Life Technologies) and placed on level 22°C surface to solidify. Cells were released by trypsinization, counted by trypan-blue exclusion test, and resuspended in 0.5% low T_m agarose (Life Technologies) in DMEM at a density of 1.0×10^6 cells/ml. 0.5 ml of this cell suspension was added to each of the Petri dishes. After cooling at 4°C for 15 minutes, DMEM containing 10% FBS, antibiotics, and 50 µg/ml ascorbic acid (Sigma) was added and the Petri dishes were transferred at 37°C in 95% humidified air and 5% CO_2. Medium was replaced every day.

In vivo assay

Skeletal precursor cells at different passages from example 3 were released by trypsin treatment, washed twice in sterile PBS, and counted by trypan-blue exclusion test. 5×10^6 cells were resuspended in a volume of 50-100 µl of PBS, and injected intramuscularly in the thigh of female, 4-5 week old immunodeficient mice. Animals were sacrificed after 3 weeks by cervical dislocation and the thigh dissected to retrieve the implant. Implants were weighed, and either snap-frozen and stored in liquid nitrogen or fixed in freshly-made 4% formaldehyde for 4 hours. After fixation the samples were included in paraffin, cut 5 µm thick sections and colored according to standard protocols (alcian blue pH 2.5, toluidin blue, Masson's trichrome, safranin O) (Manual of Histological Techniques). Figure 3 shows implants retrieved from nude mice three weeks after intramuscular injection of 5×10^6 human periost um-derived skeletal precursor cells. The Masson's trichrom (3A) and the weak alcian blue staining at pH 2.5 (3B) show a poorly differentiated fibrous tissue highly

reminiscent of periosteum. This indicates that skeletal precursor cells can grow anchorage-independently in vivo, retaining their phenotypic stability.

In situ hybridization for human-specific alu sequence was carried out on the retrieved implants for the identification of human cells as follows.

In situ hybridization for human-specific alu repeats

In situ hybridization was performed as described by Kuznetsov et al. (1997), *J. Bone Min. Res.* 12:1335-47). Figure 4 shows that the fibrocartilaginous tissue obtained from the *in vivo* assay is of human origin and not from the mouse host.

Example 6 - Forming cartilage *in vitro* with skeletal precursor cells

Induction and formation of cartilage *in vitro*, demonstrated by alcian blue staining, von Kossa method and molecular analysis for cartilage markers by RT-PCR, was achieved by micromass culture of skeletal precursor cells from example 3 at a very high cell density and treating said cells with TGF-β 1 or TGF-β 3 at a final concentration of 10 ng/ml.

Micromass culture

Expanded skeletal precursor cells from various-aged human donors at different passages from example 3 were released by trypsin treatment, counted by trypan-blue exclusion test, and resuspended in DMEM supplemented with 10% FBS and antibiotics at a cell density of 2×10^7 cells per ml. Micromass cultures were obtained by pipetting 20 µl-droplets of cell suspension into individual wells of 24-well plates. After cells were let attach without medium for 3 hours, chemically defined serum-free medium without growth factors was added. The day of introduction into micromass culture was designated as day 0. Recombinant human TGF-β 1 or TGF-β 3 (available from R & D Systems) were dissolved in 4 mM HCl containing 1 mg/ml bovine serum albumin ("BSA") and added to the culture medium at a final concentration of 10 ng/ml every day starting on day 1, when the culture medium was changed. The same amount of 4 mM HCl containing 1 mg/ml BSA was added to the culture medium as a control. Micromass cultures were harvested at different periods of time for RT-PCR analysis and alcian blue staining as explained below. For comparison, human

skin fibroblasts were cultured under identical conditions.

Alcian blue staining

Cells were rinsed twice with PBS, fixed in methanol for 1 hour at –20°C, washed with distilled water, and covered overnight with alcian blue at pH 0.2 (0.5% alcian blue available from Sigma in 1N HCl). In parallel were used human skin fibroblasts as a negative control, and human articular chondrocytes as a positive control.

Figure 5 shows alcian blue staining at pH 0.2 of human skeletal precursor cells in micromass culture either untreated (5A) or treated for 6 days with 10 ng/ml TGF-β1 (5B).

von Kossa method

It was used to detect mineral deposition. Cells were washed twice with distilled water and incubated with freshly prepared 2% silver nitrate (Sigma) in the dark for 10 minutes. Wells were rinsed 3 times with distilled water. The last aliquot of water was kept in the wells while they were exposed to bright light for 15 minutes. The reaction was stopped by thorough rinsing the wells with distilled water. As positive control human osteoblasts were used in parallel.

Alkaline phosphatase ("AP") activity

It was determined in cell lysates according to standard protocols. Briefly, cells plated in 24 well-plates were washed twice in PBS, and disrupted by sonication in 500 µl of PBS containing Triton X-100 (0.05% final concentration). Aliquots of 100 µl were assayed for enzyme activity in 0.1 M sodium barbital buffer pH 9.3 and *p*-nitrophenylphosphate (Sigma) as substrate at 37°C for 30 minutes. Released *p*-nitrophenol was converted to sodium *p*-nitrophenylate by adding 0.25 M NaOH and the amount of *p*-nitrophenylate was determined by measuring the absorbance at 400 nm. Activity was normalized to protein content measured by the Bradford protein assay using BSA as standard and expressed as units of AP activity per milligram of cell protein.

Example 7 - Enhancing cartilage forming ability of chondrocytes with skeletal precursor cells

The interactions of skeletal precursor cells and articular chondrocytes were assessed both *in vitro* and *in vivo*. For these experiments we used human

skeletal precursor cells from example 3 and pig articular chondrocytes, obtained as indicated below, in order to determine the relative contribution from the two different cell types to the cartilage forming process. As explained in example 5, the contribution of human cells can be ascertained by performing an *in situ* hybridization for human-specific alu genes. The addition of human skeletal precursor cells from example 3 to pig articular chondrocytes resulted in a dramatic impact on the cartilage forming potential of the chondrocytic cells. In particular, an increase in the amount of cartilage made and at the same time a decrease in the threshold of the *in vivo* assay (i.e. less than one million cells were required for cartilage formation and organization) were observed.

Obtaining pig articular chondrocytes

Cartilage was sliced full thickness from metatarsal and metatarso-phalangeal joints from an adult pig and placed in HBSS supplemented with antibiotics. After two washes in HBSS containing antibiotics for 5 minutes at 37°C, cartilage was finely minced and placed in a sterile 0.2% crude collagenase solution in high-glucose DMEM containing 10% FBS and antibiotics. After overnight incubation at 37°C cells were washed twice in culture medium (DMEM containing 10% FBS and antibiotics) and counted with trypan-blue exclusion test to adjust to the number of viable cells.

In vitro co-culture

Serially passaged human skeletal precursor cells from example 3 and freshly isolated pig articular chondrocytes were plated in micromass cultures, using conditions described in example 6, in the same well of a 12-well-plate, without adding growth factors. As controls, in one well were pipetted 2 micromasses of human skeletal precursor cells, and in another well 2 micromasses of pig articular chondrocytes. In each well the 2 micromasses were not in physical contact. The micromasses were harvested for histochemical analysis and staining protocols.

In vivo co-implantation

Serially passaged human skeletal precursor cells from example 3 and freshly isolated pig articular chondrocytes were intramuscularly injected into nude mice at different ratios, as indicated in table 2. As controls, human skeletal precursor cells and pig articular chondrocytes were also inject d separately,

using the same cell densities. Animals were sacrificed after 3 weeks by cervical dislocation. Implants were weighed, and either snap-frozen and stored in liquid nitrogen or fixed in freshly-made 4% formaldehyde for 4 hours. After fixation the samples were included in paraffin, cut 5 µm thick sections and colored according to standard protocols (alcian blue pH 2.5, toluidin blue, Masson's trichrome, safranin O) (Manual of Histological Techniques). *In situ* hybridization for human-specific alu sequence was performed to detect and distinguish human skeletal precursor cells from pig articular chondrocytes within the implant. Results were as follows:

Table 2

Chondrocytes	Skeletal precursor cells	Total No. of cells	Implant
5	0	5	Hyaline cartilage
0	5	5	Fibrous tissue
4	1	5	Hyaline cartilage
4	0	4	Hyaline cartilage
2.5	2.5	5	Hyaline cartilage
2.5	0	2.5	Hyaline cartilage
1	4	5	Hyaline cartilage
0.5	4.5	5	Hyaline cartilage

All numbers of cells are expressed in millions.

Although the present invention has been described with reference to skeletal precursor cells the skilled person will appreciate that the present invention may be adapted to any form of tissue repair. The method to be followed is to identify the embryonic marker or markers which identify precursor cells for the specific tissue cells to be repaired and then to select cells from the organism which exhibit the marker. The selected cells may then be expanded *ex vivo/in vitro* and subseuqntly re-implanted to repair the tissue.

CLAIMS

1. Us of an embryonic marker to identify precusor cells in a post-natal cell differentiation pathway.

2. The use according to claim 1 wherein the marker is a gene or a protein expressed by a gene in the precursor cells.

3. The use according claim 1 or 2 to wherein the the embryonic marker identifies precursor cells in skeletal development of mamals.

4. The use according to any previous claim wherein the embryonic marker identifies precursor cells belonging to a joint interface in mammals.

5. The use according to any previous claim wherein the embryonic marker is a cartilage-derived morphogenetic protein CDMP-1 or a transforming growth factor β having at least 80% homology with CDMP-1 as a marker of skeletal precursor cells from any part of a mammalian body.

6. Use of reagents and/or antibodies recognizing specific cell markers for sorting, out of the precursor cells marked according to any of the claims 1 to 5, cell subpopulations for further processing by *in vitro*/*ex vivo* treatment in specific lineages.

7. Use of reagents and/or antibodies recognizing cell surface markers for sorting and enriching, out of the precursor cells marked according to any of the claims 1 to 5, a subpopulation of chondrogenic cells.

8. Use of skeletal precursor cells marked according to any of the claims 1 to 5 for producing or repairing connective tissue in a mammal.

9. Use according to claim 8, wherein the said cells are cultured at a cell density of at least 10^5 cells/ml.

10. Use according to claim 8 or claim 9, comprising further administration of a factor that stimulates differentiation of the skeletal precursor cells into the type of connective tissue to be produced or repaired.

11. Use of skeletal precursor cells marked according to any of claims 1 to 5 as a source of growth factors.

12. Use of skeletal precursor cells marked according to any of claims 1 to 5 as matrix producing cells.

13. Use according to claim 12, wherein the said matrix further comprises a bio-resorbable polymer.

14. Use according to claim 12 or claim 13 for the treatment of subglottic stenosis, tracheomalacia, chondromalacia patellae, osteoarthritis and traumatic lesions in a mammal.

15. A procedure for joint surface defect repair in a mammal comprising the co-implantation of skeletal precursor cells marked according to any of claims 3 to 5 and chondrocytes.

16. A method for enhancing the implantation of a prosthetic device in connective tissue comprising the step of implanting a prosthetic device having precursor cells adhered thereto under conditions suitable for differentiating the cells into the connective tissue desired.

17. A method for storing precursor cells marked according to any of claims 1 to 5, comprising storage conditions including a storage temperature below –100°C.

ABSTRACT

The us of an embryonic marker to identify precusor cells in a post-natal differentiation pathway is described. The marker may be a gene or a protein expressed by a gene. For example, a cartilage-derived morphogenetic protein CDMP-1 or a transforming growth factor β having at least 80% homology with CDMP-1 is described as a marker of skeletal precursor cells from any part of a mammalian body.

Figure 1

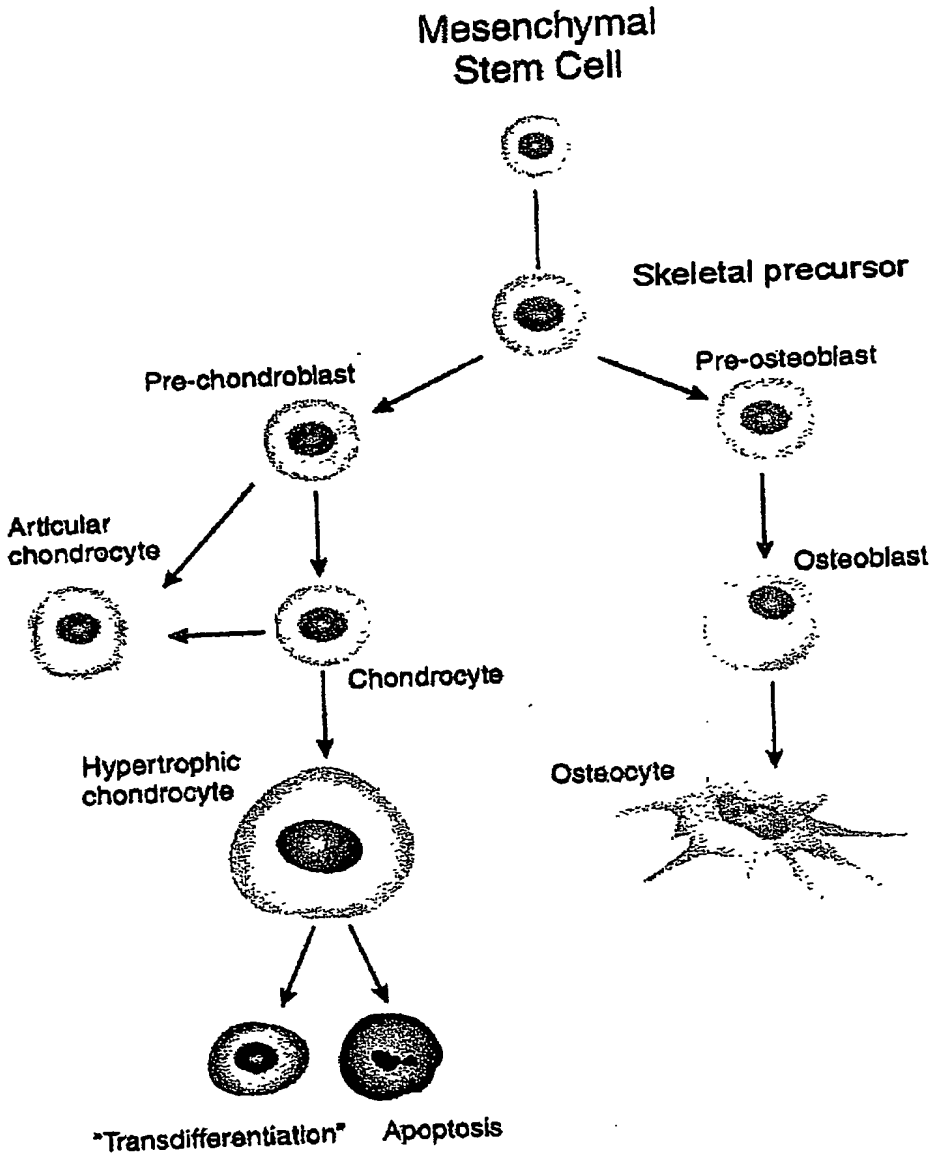

Mesenchymal Stem Cell
Skeletal precursor
Pre-chondroblast
Pre-osteoblast
Articular chondrocyte
Osteoblast
Chondrocyte
Hypertrophic chondrocyte
Osteocyte
"Transdifferentiation"
Apoptosis

Figure 2A

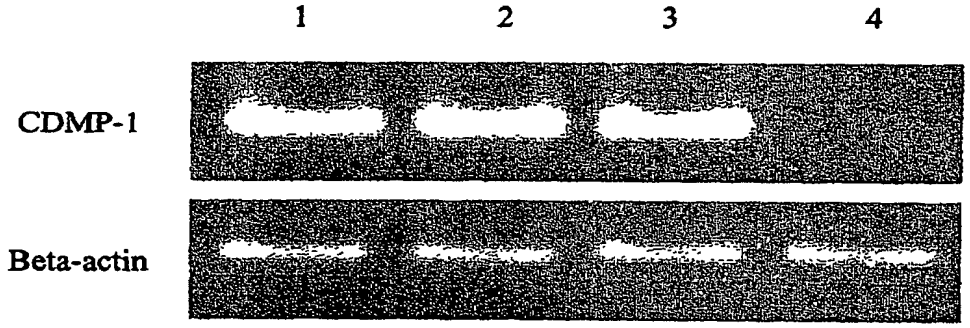
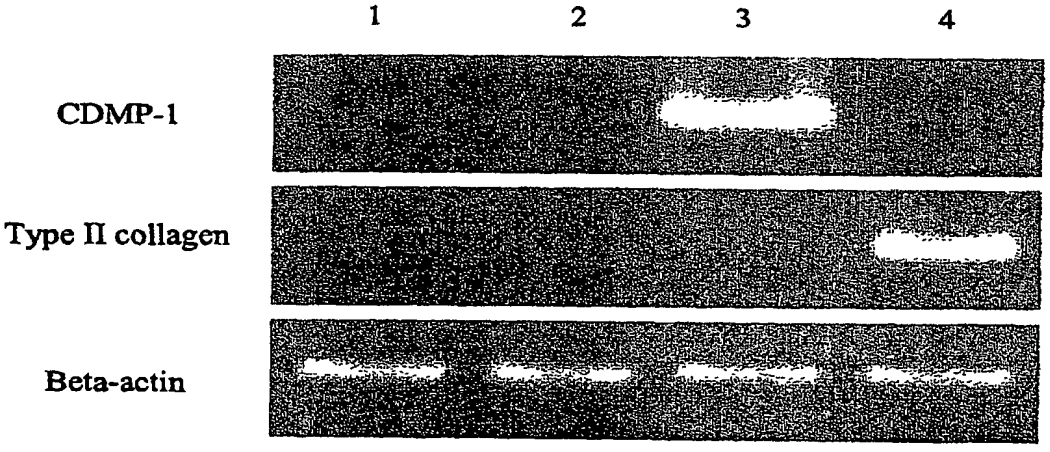


Figure 2B



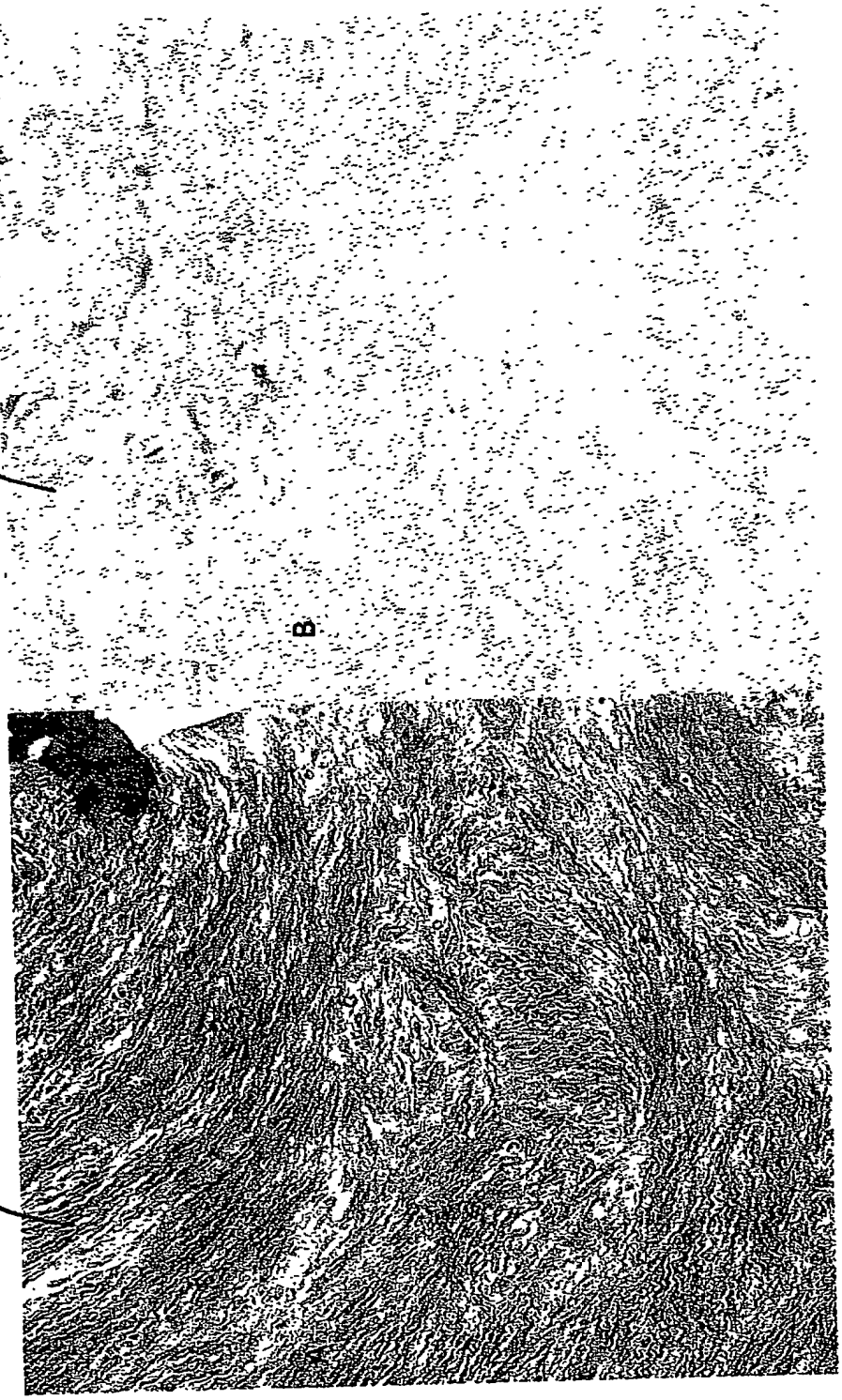


Figure 3

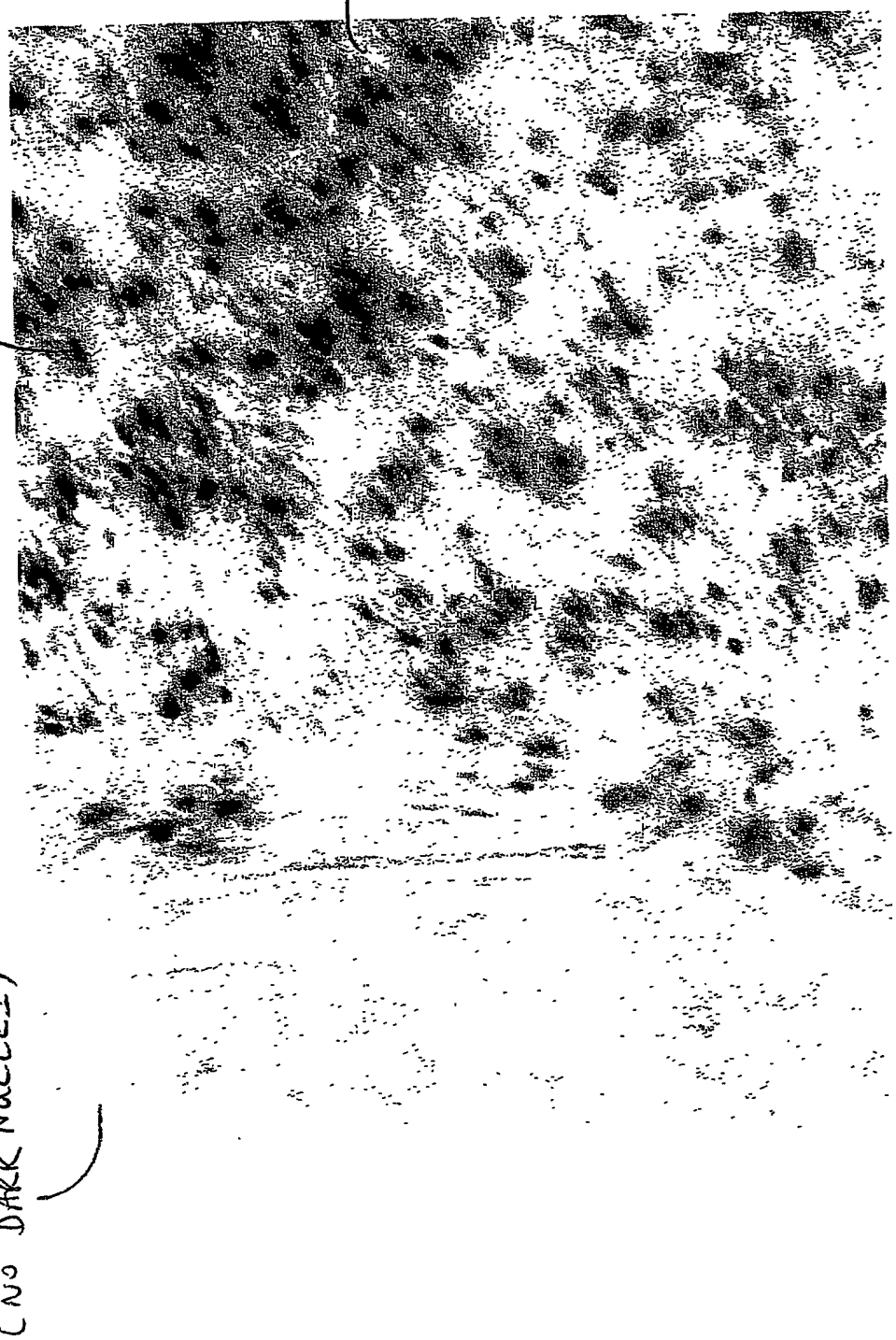

HUMAN TISSUE
NUCLEI STAINED DARKLY
MOUSE TISSUE (NO DARK NUCLEI)

Figure 4


STRONG ALCIAN BLUE STAIN
NO BLUE COLOUR DESPITE ALCIAN BLUE STAIN
B
A

Figure 5